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THE ASSEMBLY OF CD1e IS CONTROLLED BY AN N-TERMINAL PROPEPTIDE WHICH IS PROCESSED IN ENDOSOMAL COMPARTMENTS

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Abbreviations used : immature, iDCs, and mature, mDCs, dendritic cells; membrane-associated, mCD1e, and soluble CD1e, sCD1e; phosphatidylinositol hexa- or di-mannoside, PIM_6 or PIM_2; recombinant soluble, rs; endoglycosidase, Endo H ; Peptide: N-glycosidase F, PNGase F.

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Summary

CD1e displays unique features in comparison to other CD1 proteins. CD1e accumulates in Golgi compartments of immature dendritic cells and is directly transported to lysosomes, where it is cleaved in a soluble form. In these latter compartments, CD1e participates in the processing of glycolipid antigens. Here we show that the N-terminal end of the membrane-associated molecule begins at aminoacid 20, while the soluble molecule consists of aminoacids 32 to 333. Thus, immature CD1e includes an N-terminal propeptide which is cleaved in acidic compartment and so, absent on the mature endosomal form. Mutagenesis experiments demonstrated that the propeptide controls the assembly of the CD1e α chain with β2-microglobulin while propeptide-deleted CD1e molecules are immunologically active. Comparison of CD1e cDNAs from different mammalian species indicates that the CD1e propeptide is conserved during evolution, suggesting that it may also optimize the generation of CD1e molecules in other species.

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Introduction

CD1 molecules are non classical HLA class I molecules, among which human CD1e displays several distinct features in its trafficking, function and primary structure. Human CD1a, b, c and d cycle between the plasma membrane and different endosomal compartments, and are involved in the presentation of glycolipids to T cells [1]. Conversely, CD1e initially accumulates in the Golgi compartments of immature dendritic cells (iDCs) or transfected cells as a membrane-associated form (mCD1e) and is subsequently directly transported to early and then late endosomes, where it is cleaved into a soluble form (sCD1e) [2, 3]. This form facilitates the processing of glycolipids presented by other CD1 molecules transiting through the same compartments. For example, presentation of phosphatidylinositol hexamannoside (PIM_6) to CD1b-restricted T cell clones is CD1e-dependent, whereas the presentation of dimannoside (PIM_2) is not [4]. *In vitro* experiments have shown that α-mannosidase digests PIM_6 into the immunogenic PIM_2 form, only in the presence of sCD1e [4].
Alignment of the mature α chains of human CD1 proteins, without the signal peptide, suggests that CD1e has an N-terminal extension of 8-10 aminoacids (Table I, panel A). The assumption that this extension is present on endosomal CD1e is however not supported by the N-terminal aminoacid sequence of a sheep thymic CD1 protein [5] which should correspond to endosomal sCD1e on the basis of its sequence homologies and biochemical properties. This latter sequence suggests that the 12 N-terminal aminoacids of human CD1e are cleaved. As the gene sequence of sheep CD1e is not known, it is not possible to confirm N-terminus processing of CD1e in this animal.
Comparison of CD1e proteins in different species suggests the existence of an N-terminal extension conserved during evolution (Table I, panel B), which is cleaved during CD1e maturation. The aim of the present study was to validate this hypothesis, to identify the cellular compartments where this N-terminal extension is cleaved and to elucidate its function.

Experimental

Genetic constructions

CD1e forms used in this work were derived from the cDNA encoding the isoform with the long cytoplasmic tail (CD1eL, [2]). Propeptide-deleted molecules were derived by PCR mutagenesis of cDNAs encoding CD1e. Various CD1e-derived molecules were expressed in the pEGFP-N3 vector (Clontech-Takara Bio Europe, Saint Germain en Laye, France)

Antibodies.

The anti-CD1e mAbs VIIC7, 2.9 and 20.6 have been described previously [2]. The anti-β2m mAb B1G6 was purchased from Beckman-Coulter (Villepinte, France). Polyclonal Abs specific for the N-terminus of neosynthesized CD1e were raised by immunizing rabbits with the synthetic peptide APQALQSYHLAAEEC (NeoMPS, Strasbourg, France) coupled to keyhole limpet hemocyanin. Anti-peptide Abs were immunopurified from 5 ml of antiserum by adsorption onto 1 mg of peptide coupled to NHS-activated Sepharose (GE Healthcare, Orsay, France). CD1e molecules were immunopurified with anti-CD1e mAbs (clone 20.6) chemically coupled with dimethyl pimelimidate (Perbio Science, Brebière, France) on protein A Sepharose (GE Healthcare).

Transfected cells and biochemical analyses

Transfected M10 cells expressing CD1e and CD1b molecules, alone or together, have been previously described [2, 4]. Mutated forms of CD1e were expressed in M10 cells, expressing or not CD1b, after transfection with Fugene 6 reagent (Roche Applied Science) followed by

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selection in the presence of 500 µg/ml G418 (Invitrogen, Cergy Pontoise, France). Clones expressing CD1e were identified by immunofluorescence of fixed permeabilized cells stained with the mAb 20.6, respectively, as described [2].
In western blotting analyses, cells were recovered from a 75 cm^2 flask, resuspended in lysis buffer (150 mM NaCl, 20 mM Tris pH 7.5, containing 1% Triton X100 and Complete™ protease inhibitor cocktail, Roche Applied Science) and centrifuged at 6000g to eliminate the nuclei. CD1e molecules were immunoprecipitated for 2 h using 50 µl of either VIIC7 or 20.6 hybridoma cell culture supernatant and adsorbed onto protein A Sepharose. After extensive washing, proteins were eluted from the matrix with 0.5% SDS and treated or not with endoglycosidase (Endo) H or Peptide: N-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA). The proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with the mAb VIIC7 or anti-A15C polyclonal Abs, followed by HRP-conjugated goat anti-mouse or anti-rabbit Abs, respectively. Bands were revealed with SuperSignal West Pico reagent (Perbio Science). Pulse-chase metabolic labeling, immunoprecipitations and Endo H or F treatment were carried out as previously described [2]. Molecular mass standards used were either prestained protein markers (New England Biolabs) (47.5, 32.5 and 25 kD) or 14C-labeled GE-Healthcare (45 and 30 kD).

Purification of CD1e molecules from transfected cells
Membrane and cytoplasmic proteins from $7x10^8$ stably transfected cells were extracted in 30 ml of lysis buffer (Tris 20mM, NaCl 150mM, 1% Triton X100 and protease inhibitors, pH 7.5), pre-immunoadsorbed on protein A Sepharose overnight and then incubated for 2h with 40 µg of 20.6 covalently coupled to protein A Sepharose. After extensive washing, the immunoadsorbed proteins were eluted, separated by SDS-PAGE and revealed with Coomassie blue G250.

Mass spectrometry of tryptic digests
Immunopurified recombinant CD1e molecules were separated on SDS-PAGE and stained with Coomassie Blue. As a negative control, identical procedure was performed using untransfected cells. Major species specific for transfected cell extracts, corresponding to mCD1e and sCD1e, were cut. Gel slices were washed twice with 50 µl of 25 mM NH_4HCO_3 followed by 50µl of acetonitrile. Cysteine residues were reduced by 50µl of 10 mM dithiothreitol at 57°C and alkylated by 50 µl of 55 mM iodoacetamide. After dehydratation with acetonitrile, the proteins were cleaved in gel with a 12.5 ng/µl solution of modified trypsin (Promega, Madison, WI, USA) in 25 mM NH_4HCO_3. The resulting tryptic peptides were extracted with 60% acetonitrile in 5% formic acid and analyzed by LC-MS/MS.
Measurements by MALDI mass spectrometry were carried out on an Ultraflex TOF/TOF (Bruker, Germany) mass spectrometer in reflectron mode with positive ion spectra. The samples were prepared by standard dried droplet preparation on stainless steel MALDI targets using α-cyano-4-hydroxycinnamic acid as matrix. External calibration of MALDI mass spectra was carried using singly monoisotopic peaks of a mixture of bradykinin 1-7 (m/z=757.400), human angiotensin II (m/z=1046.542), human angiotensin I (m/z=1296.685), substance P (m/z=1347.735), bombesin (m/z=1619.822), rennin(m/z=1758.933), ACTH 1-17 (m/z=2093.087) and ACTH 18-39 (m/z=2465.199). To achieve mass accuracy, internal calibration was performed with trypic peptides coming from autolysis of trypsin, with monoisotopic masses automatically annoted using Flexanalysis 2.0.
For nano-HPLC, a CapLC system (Micromass Ltd., Manchester, UK) was used. The samples were concentrated on a precolumn, and the peptides were separated on a 15 cm × 75 µm internal diameter column packed with 3 µm 100 Å C18 PepMap (LC-Packings). The MS and

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MS/MS analyses were performed with a Q-TOF 2 hybrid quadrupole/time-of-flight mass spectrometer (Micromass Ltd., Manchester, UK) equipped with a Z-spray ion source.
Mass data collected during LC-MS/MS analysis was processed and converted into a PKL file to be submitted to the search software Mascot (Matrix Science Ltd., London, UK). Searches were performed with a tolerance on mass measurement of 0.25 Da in MS and MS/MS mode in NCBI (The National Center for Biotechnology Information) database.

Production of recombinant soluble (rs) CD1e in insect cells
The luminal domains of CD1e without the signal peptide, beginning at Ala 20 (rsCD1e) or Glu 32 (rsΔ12CD1e) and ending at Ser 305, were directly fused to the signal peptide of BIP and a C-terminal V5His tag (pMTbipV5HisA, Invitrogen). The two forms were expressed in S2 Drosphila cells and purified from culture medium as previously described [4, 6]. The resulting preparations included only the CD1e α chain and β2-m, as shown by SDS-PAGE analysis. N-terminal aminoacid sequencing confirmed that rsCD1e and rsΔ12CD1e began with the expected aminoacid sequence APQALQ or EEQLSF, respectively (data not shown).

Antigen presentation assays
Transfected M10 cells ($3x10^4$/well) growing in RPMI-1640 medium supplemented with 10% FCS, 10 mM HEPES, 2 mM Ultraglutamine II, MEM nonessential amino acids, 1 mM sodium pyruvate (all from Lonza, Brussels, Belgium) and 100 µg/ml kanamycin (Invitrogen, Basel, Switzerland) were preincubated for 2 h at 37°C with different concentrations of purified PIM_6 [7, 8], or GM1 ganglioside (Matreya, Pleasant Gap, PA), before addition of T cells (10^5/well in triplicates). The T cells used were the CD1b-restricted, PIM_6-specific and CD1e-dependent clone DL15A30, and the CD1b-restricted and GM1-specific clone GG33A [9], which is CD1e-independent [4]. Supernatants were harvested after 36 h of incubation, and cytokine release was measured with an enzyme-linked immunosorbent assay for GM-CSF or IFN-γ (ELISA) kit (R&D Systems, Abingdon, UK). Data were expressed as mean arbitrary units (A.U. for GM-CSF) or pg/ml (for IFN-γ) ± the standard deviation (SD) of triplicates.
The biological activity of rsCD1e was also tested by pre-incubating overnight CD1b-transfected M10 cells with 20 µg/ml rsCD1e. Cells were then washed and processed for antigen presentation as described above.

Enzymatic digestion of PIM_6.
Purified PIM_6 (100 µg) was sonicated in 50µl of mannosidase buffer (sodium acetate 0.1 M, $ZnSO_4$ 1 mM pH 4.5). RsCD1e (4.3 nanomoles in 150 µl) was equilibrated by dialysis against mannosidase buffer for 1h and then incubated with PIM_6 (100 µg in 50 µl) and with jack bean α-mannosidase (20 µl, 1.8 mg/ml, Sigma) for 1 h at 37 °C. Glycolipids were extracted and analyzed by mass spectrometry as previously described [4].

Results
Characterization of the ends of CD1e forms provides evidence for processing of the N-terminus
Analysis of pre-CD1e sequence predicts that cleavage of the signal peptide occurs between Ala19 and Ala20 (Table IA), whereas data from a CD1e-like sheep protein indicate that the 12 following N-terminal aminoacids have been removed from the lysosomal form. To confirm these predictions, CD1e molecules from transfected M10 cells were immunopurified and separated by SDS-PAGE. After Coomassie blue staining, two protein species were recovered having apparent molecular masses corresponding to those of mCD1e and sCD1e (Fig. 1A). Comparison of the MALDI-TOF mass spectra of the tryptic digests of the two forms of CD1e revealed four peaks of m/z 1050.54, 1300.69, 1790.89 and 2159.08 Da,

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specific to the 45 kD mCD1e form (Fig. 1B). These peaks were not predicted by *in silico* trypsin digestion of mCD1e and hence probably corresponded to semi-tryptic species (with only one trypsin-generated end) resulting from digestion of the N- and C- terminal ends of mCD1e. On the basis of this hypothesis, the m/z value of the predominant 2159.08 Da peak corresponded to the theoretical m/z value of the peptide APQALQSYHLAAEEQLSFR, generated by trypsin digestion of the predicted N-terminal end of mCD1e (Table IA). The three other peaks represented shorter N-terminal ends (Table II). The identities of these peptides were confirmed by *de novo* sequencing after LC-MS/MS (Fig. 1C).
A similar analysis of the tryptic digest of sCD1e revealed three prominent peaks of m/z 908.45, m/z 1585.80 and m/z 1913.00 Da (Fig. 1B). LC-MS/MS showed that the 1913.00 Da peak corresponded to the internal tryptic peptide NLQSLFQLYFHSFIR (theoretical molecular mass 1912.00), which represents aminoacids 92-106 of preCD1e (data not shown). Since a glycosylation site is predicted at Asn 84, it is possible that the processing of carbohydrates in lysosomes results in a greater accessibility to trypsin of the aminoester bond between aminoacids 91 and 92, which would explain why this peak was only observed in the tryptic digest of sCD1e. The two first peaks were not predicted by *in silico* trypsin digestion of CD1e and thus might result from digestion of the N- or C- terminal end of sCD1e. This conclusion was confirmed by *de novo* sequencing after LC-MS/MS (Fig.1C, Table II). The m/z 908.45 Da species corresponded to a peptide derived by proteolysis of the N-terminal end of sCD1e, EEQLSFR, resulting from the cleavage of the 12 N-terminal aminoacidsof mCD1e. The aminoacid sequence of the m/z 1585.80 Da entity was HSSLGGHDLIIHWGG (theoretical m/z 1585.78 Da) and hence corresponded to the C-terminal end of sCD1e. This determination confirmed that sCD1e is generated by a proteolytic cleavage of the luminal part of mCD1e, near the transmembrane domain.
Thus, newly synthesized CD1e molecules include a 12-aminoacid N-terminal extension, hereafter called the propeptide, which is absent from lysosomal sCD1e. Subsequent experiments were designed to identify the cellular compartment where the propeptide is cleaved.

The 12 N-terminal aminoacids of CD1e are cleaved in endosomes

To confirm that these 12 aminoacids are present on ER-localized CD1e molecules, polyclonal antibodies against a synthetic peptide including the 12 N-terminal aminoacids of CD1e (A15C) were raised in rabbits. CD1e was immunoprecipitated from extracts of transfected M10 cells expressing CD1e using the mAb VIIC7. This mAb recognizes the cytoplasmic domain of CD1e and in transfected M10 cells immunoprecipitates molecules which are EndoH sensitive and thus located in the ER [2]. The immunoadsorbed CD1e was then analyzed by western blotting using either VIIC7 or the polyclonal anti-A15C Abs (Fig. 2). As compared to VIIC7 mAb, A15C Ab poorly detected CD1e molecules, which may explain why it only revealed EndoH-sensitive CD1e molecules immunoprecipitated with the mAb VIIC7. Nevertheless, these experiments demonstrated that the anti-propeptide Abs recognized EndoH sensitive, ER-localized, CD1e molecules.
To confirm that the N-terminal extension is missing on lysosomal sCD1e and to identify the cellular compartment in which it is processed, a CD1e form deleted of its 12 N-terminal aminoacids (Δ12CD1e) was expressed in M10 cells. Confocal immunofluorescence microscopy showed that CD1e and Δ12CD1e have a similar cellular distribution, accumulating in TGN46^{+} compartments of the Trans Golgi Network and CD63^{+} lysosomal compartments (supplementary data 1). Then, the biochemical maturation of Δ12CD1e was compared to that of complete CD1e by pulse-chase labeling experiments, and immunoprecipitation with the mAb 20.6. This mAb immunoprecipitates Golgi- and

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endosome-localized CD1e molecules as well as a small percentage of molecules in the ER [2]. Immediately after the pulse and during chase, the mCD1e and mΔ12CD1e forms displayed the expected small differences in electrophoretic mobilities (Fig. 3A, bands m). In contrast, after 2 h of chase, the cleaved molecules (Fig. 3A, bands s) had the same electrophoretic mobility, demonstrating the absence of the 12 N-terminal aminoacids on lysosomal CD1e.

Thus, after 4 hours of chase, the N-terminal extension is still present on mCD1e molecules, which are Endo H-resistant and thus are transiting, or have transited, through the Golgi apparatus [2] (see also Fig. 4). Thus, likely, the propeptide is cleaved in a post-Golgi compartment. To further investigate this mechanism of CD1e processing, transfected cells expressing CD1e or Δ12CD1e were pulse labeled and chased for 4 h in the presence of bafilomycin, after which CD1e was immunoprecipitated with the mAb 20.6, deglycosylated and analyzed by SDS-PAGE (Fig. 3B). The electrophoretic mobilities of mCD1e and mΔ12CD1e remained constant throughout the 4 h of chase in the presence of bafilomycin. Within 4 h of chase in the absence of bafilomycin, the majority of full length CD1e molecules (i.e. with propeptide) leave the Golgi compartments, reach late endosomes and become fully processed ([2], see also Fig. 4)

Since bafilomycin blocks the endosome acidification and transport from late endosomes to lysosomes [10], but not transport from Golgi compartments to endosomes, the full length CD1e molecules immunoprecipitated after 4h of chase in the presence of bafilomycin were mostly derived from late endosomes. Thus, bafilomycin blocks both the generation of sCD1e, as already reported [2], and cleavage of its 12 N-terminal residues. We previously found that a 27 kD protein co-immunoprecipitates with CD1e from cell extracts of bafilomycin-treated cells [2, 11]. Since this protein is PNGase resistant, it is unrelated to CD1e. Metabolic experiments here demonstrate that this interaction is preserved after deletion of the propeptide. These experiments demonstrate that the CD1e propeptide is cleaved by pH dependent mechanism in late endosomal compartments.

The propeptide controls the exit of CD1e from the ER

Propeptides have very diverse functions. They can be involved, for example, in controlling the ER exit of proteins (as in the case of proalbumin, apolipoprotein A-I or ADAMTS9), in post-translational modifications (vitamin K-dependent blood clotting factors), or in regulating the activity of pro-proteases. To investigate whether maturation of the CD1e protein is controlled by the propeptide, we performed semi-quantitative biochemical studies using pulse-chase labeling followed by immunoprecipitation with different anti-CD1e mAbs. Immunoprecipitation with the mAb VIIC7 (Fig. 4A), which recognizes all mCD1e molecules, even those which are not assembled with β2m, revealed no major differences between CD1e and Δ12CD1e forms. Densitometry scanning showed that during chase the amounts of immunoprecipitated CD1e and Δ12CD1e decreased with similar kinetics (data not shown).

Interestingly, immunoprecipitation with the conformation-dependent mAb 20.6 gave more informative results since the kinetics of the generation of soluble Δ12CD1e were significantly slower than for soluble wild type CD1e (Fig. 4A-C). Moreover, the generation of EndoH-resistant Δ12CD1e molecules, ie molecules reaching the Golgi compartments, appeared to be delayed throughout the 8 hours of chase. In particular, EndoH sensitive mΔ12CD1e molecules were still detected at the end of the chase (Fig. 4A, bands m, Endo H-treated samples). Hence Δ12CD1e molecules exit the ER more slowly from the ER than wild type CD1e molecules.

This last finding suggests that the propeptide might control the assembly of β2m/CD1e α chain heterodimers, and/or their exit from the ER. To discriminate between these two possibilities, transfected cells expressing complete or propeptide-deleted CD1e molecules

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were pulse-labeled and chased for different periods of time (Fig. 4B). After immunodepletion with the mAb W6/32, CD1e molecules were immunoprecipitated using the mAb 20.6 or B1G6, which recognize the CD1e α chain or β2m, respectively. The immunodepletion of HLA class I molecules was efficient since relatively small amounts of these molecules were immunoprecipitated with the mAb B1G6. Nevertheless, the presence of HLA α chains in 20.6 immunoprecipitates suggests that these species resulted from incomplete immunoadsorption of W6/32-HLA class I immune complexes, despite two additional runs of protein A sepharose adsorption. Glycosylated CD1e and HLA class I α chains migrated at similar positions, but the two species were well resolved after deglycosylation. At all time points, after immunoprecipitation with the mAb 20.6, the ratio of sΔ12CD1e to mΔ12CD1e molecules was less than for complete CD1e (Fig. 4C). In contrast, after anti-β2m immunoprecipitation, the ratios were equivalent for CD1e and Δ12CD1e after 2 or 4 h of chase (Fig. 4C). At 8 h of chase, an unrelated protein was detected migrating as CD1e α chain and prevented clear interpretation.

Taken together, these data suggest that the absence of the propeptide leads to delayed assembly of CD1e α chain with β2m, and consequently retards the generation of soluble CD1e.

Propeptide deleted molecules are active

CD1e is necessary for the presentation of the mycobacterial PIM_6 antigen by CD1b to specific T cells and for the *in vitro* conversion of PIM_6 into PIM_2 by α-mannosidase [4]. We used this antigen processing and presentation model to investigate whether the propeptide is required for proper CD1e function. M10 cells expressing CD1b alone, or co-expressing CD1b together with CD1e or Δ12CD1e, were incubated with PIM_6 and then used to stimulate CD1b-restricted PIM-specific T cells. The double transfected M10 cell expressing CD1b and either CD1e or Δ12CD1e presented PIM_6 with similar efficiency thus showing that Δ12CD1e is able to assist processing and presentation of this lipid antigen (Fig. 5A). In control experiments, transfected M10 cells only expressing CD1b did not present PIM_6, confirming the requirement of CD1e for presentation of this antigen. In addition, the CD1b-restricted presentation of GM1 ganglioside, a CD1e-independent antigen, was not influenced by either form of CD1e (Fig. 5B).

The influence of the propeptide on the CD1e chaperone function was also investigated using two recombinant soluble CD1e molecules. M10 cells expressing CD1b only were pulsed overnight with rsCD1e or rsΔ12CD1e in order to promote their internalization, then washed and incubated with PIM_6, together with specific T cells. The two forms of rsCD1e molecules were equally active in assisting PIM_6, presentation, confirming that both forms have similar behavior in assisting PIM_6 processing and presentation (Fig. 5C). Finally, we looked at the *in vitro* digestion of PIM_6 by α-mannosidase. PIM_6 was incubated with α-mannosidase together with rsCD1e or rsΔ12CD1e and digestion products were analyzed by mass spectrometry. Both CD1e molecules facilitated PIM digestion by α-mannosidase as shown by appearance of PIM_2 (Fig. 5D).

Taken together, these experiments indicate that the absence of the propeptide delays the assembly and maturation of CD1e but does not alter its immunological function.

Discussion

CD1e is a unique molecule among the CD1 family members. It is the only CD1 protein not expressed on the cell surface, and it is the only protein to be converted into a soluble lysosomal form. Its immunological relevance has recently been identified [4], however, it remains unclear how it matures and becomes immunologically functional. In

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order to better characterize the biochemical maturation of CD1e, we determined the C-terminal sequence of lysosomal sCD1e molecules and compared the N-terminal sequences of the soluble and membrane-associated forms by analyzing their tryptic digests by mass spectrometry. These analyses confirmed that sCD1e is generated by cleavage of the luminal part of the molecule, proximal to the transmembrane domain. Several peaks of the of tryptic digest of mCD1e and corresponding to the N-terminal sequences were identified. The major one was deduced by signal peptide prediction algorithm, while the shorter ones may result from processing by ER and/or Golgi-localized protease(s) or from proteolytic events during the purification of CD1e. The major finding of our study was that CD1e is synthesized as a precursor bearing an N-terminal propeptide of 12 aminoacids. This propeptide is processed within endosomes and occurs nearly concomitantly with the generation soluble CD1e. In addition, preliminary data suggest that the two processes are independent.

Pulse chase labeling experiments demonstrated that in contrast to wild type CD1e, more Endo H-sensitive Δ12CD1e molecules were immunoprecipitated by the conformation-dependent mAb 20.6 at all time points. Similar results were obtained when the mAb 2.9 was used in immunoprecipitation experiments (data not shown). In contrast, the kinetics of maturation of CD1e and Δ12CD1e molecules immunoprecipitated with an anti-β2m mAb were comparable. This strongly suggests that the exit of CD1e/β2m complexes from the ER does not depend on the propeptide and that the latter mainly facilitates their assembly. A requirement for propeptides in the secretion of other proproteins has been described and one important issue is how they influence ER retention. The glycosylation of three asparagines in its 287 aminoacid long propeptide controls secretion ADAMTS9 metalloprotease [12]. Apolipoprotein A-I is synthesized with a 6-aminoacid propeptide and accumulates in Golgi compartments before being secreted. Deletion of the propeptide leads to partial retention of apolipoprotein A-I in the ER and to its almost complete disappearance in other cellular compartments [13]. Secretion of rat proalbumin is also controlled by a propeptide, since its deletion induces ER retention and very poor protein release [14]. How the short propeptides influence the secretion of these proteins has not been elucidated.

While CD1e propeptide facilitates CD1e assembly, propeptide-deleted CD1e molecules are immunologically functional. Indeed, cells expressing Δ12CD1e, which lacks the propeptide, presented the CD1e-dependent PIM_6 antigen with similar efficiency as cells expressing intact CD1e. Furthermore, rsΔ12CD1e produced in insect cells was able to complement M10 cells only expressing CD1b for the presentation of PIM6, like rsCD1e molecules [4]. Finally, in vitro, the two forms of recombinant soluble CD1e similarly facilitated complete digestion of PIM_6 by α-mannosidase.

In conclusion, our studies demonstrate that the CD1e propeptide facilitates the assembly of CD1e in the ER, and consequently its egress and transport to Golgi compartments. This is conserved in all species in which the CD1e cDNA has been characterized, thus suggesting that it may optimize the generation of CD1e also in other species. It remains to be elucidated why only CD1e displays such a property among CD1 molecules and other members of the MHC class I family.

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Tables and Figures

FIGURE 1. Characterization of the N- and C-terminal ends of lysosomal sCD1e. CD1e molecules from solubilized extracts of $7x10^8$ transfected M10 cells were immunopurified on immobilized 20.6. After separation by SDS-PAGE, proteins were revealed with Coomassie blue (*A*). Bands corresponding to membrane-associated (m) and soluble (s) CD1e were recovered, digested with trypsin and analyzed by MALDI mass spectrometry. *B*). Comparison of the two spectra revealed identities specific to mCD1e (Δ) and sCD1e (*). *C*) *De novo* sequencing after LC-MS/MS of different precursor ions (i) doubly charged m/z 454.7 (ii) triply charged m/z 529.2 and (iii) triply charged m/z 720.3 confirmed the peptide sequences as (i) EEQLSFR attributed to the N-terminus of sCD1e, (ii) HSSLGGHDLIIHWGG attributed to the C-terminus of the sCD1e and (iii) APQALQSYHLAAEEQLSFR attributed to the N-terminus of mCD1e. The y- and b- ion peaks derived from internal fragmentation of peptide bonds provide amino acid sequence information read from the C-terminus and N-terminus of the peptide, respectively.

FIGURE 2. Evidence for an N-terminal extension on CD1e. Untransfected and CD1e-transfected M10 cells were lysed and CD1e was immunoprecipitated with the mAb VIIC7. The immunoprecipitated proteins were left untreated (U) or deglycosylated with Endo H (H) or PNGase F (F) and then analyzed by Western blotting using the mAb VIIC7 or the polyclonal antibody A15C recognizing the 12-aminoacid N-terminal peptide. Upper panels, transfected cells; lower panels, untransfected cells.

FIGURE 3. The CD1e propeptide is only present on membrane-associated molecules and is cleaved in late endosomal compartments. Transfected M10 cells expressing complete CD1e (C) or the propeptide-deleted CD1e mutant (Δ) were metabolically labeled with ^{35}S methionine and cysteine and recovered immediately *A*) or chased for 2 or 4h in the presence of bafilomycin (*B*). The cells were then lysed and CD1e was immunoprecipitated with 20.6. Samples were left untreated (U) or deglycosylated with PNGase F (F). Bands m and s correspond to mCD1e and endosomal sCD1e, respectively. Band p corresponds to a non N-glycosylated 27 kD protein which co-immunoprecipitates with CD1e after bafilomycin treatment.

FIGURE 4. The biochemical maturation of CD1e is delayed in the absence of the propeptide. *A*) Transfected M10 cells expressing CD1e or Δ12CD1e were pulse labeled and chased for 0, 2, 4 or 8h. CD1e molecules were recovered from detergent solubilized cell extracts using the mAb VIIC7 or 20.6 and protein A-Sepharose. The immunoadsorbed molecules were left untreated (U) or digested with Endo H (H) or PNGase F (F) and separated by SDS-PAGE. Gels were exposed for autoradiography for 11 days (VIIC7) or 23 days (20.6). *B*) Untransfected M10 cells (M10) and transfected cells expressing complete (CD1e) or propeptide-deleted CD1e (Δ12CD1e) molecules were pulse labeled and then chased for 0, 2, 4 or 8 h. After immuno-depletion of HLA class I molecules with the mAb W6/32, CD1e molecules were immunoprecipitated with 20.6 or the anti-β2m mAb B1G6 then treated with Endo H (H) or PNGase F (F) or not treated (U), separated by SDS-PAGE and revealed by autoradiography. *C*) The autoradiograms from two pulse-chase labeling experiments using immunoprecipitation with 20.6 [see Fig 4A (1) and 4B (2)] or B1G6 [see Fig4B (2) and an additional experiment (data not shown, 1)] were analyzed by densitometry scanning of PNGase F-treated samples. At each time point and for wild type CD1e (C) or Δ12CD1e (Δ), the percentage of soluble among total immunoprecipitated CD1e α chains was determined.

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FIGURE 5. The Δ12CD1e form is immunologically active. *A*) Transfected M10 cells expressing CD1b alone (■) or co-expressing CD1b together with CD1e (○) or with Δ12CD1e (□) were incubated with PIM_6 (A) or GM1 (*B*) for 4 h before addition of specific T cell clones DL15A30 or GG33a, respectively. GM-CSF release was used as a read-out of T cell activation. *C*) In another type of experiment, M10 cells expressing CD1b alone were incubated with 20 µg/ml rsCD1e (○) or rsΔ12CD1e (□) or in the absence of rsCD1e (■). After overnight incubation, cells were washed and incubated with PIM_6 and specific T cells. IFNγ release was used as a read-out of T cell response. *D*) PIM_6 [4] was digested with α-mannosidase (α-m) in the presence of rsCD1e, rsΔ12CD1e or in their absence. The relative intensities of the peaks, corresponding to the different PIM species generated by digestion were measured by MALDI-MS mass spectrometry.

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TABLE I

Predicted N-terminal aminoacid sequences of human CD1 proteins and mammalian CD1e molecules after cleavage of their signal peptide

	Proteins	Predicted N-terminal sequences[a]
	Human CD1a	DGLKEPLSFHVTWIASFYNHSWKQNLVSGW
	Human CD1b	EHAFQGPTSFHVIQTSSFTNSTWAQTQGSGW
A	Human CD1c	DASQEHVSFHVIQIFSFVNQSWARGQGSGW
	Human CD1d	EVPQRLFPLRCLQISSFANSSWTRTDGLAW
	Human CD1e	**APQALQSYHLAA**EEQLSFRMLQTSSFANHSWAHSEGSGW
	Human CD1e	**APQALQSYHLAA**EEQLS
	Macaque CD1e	APQALQSYRPAAEDPLS
B	Guinea-Pig CD1e	GPQPLGWHHPAEAEEPLI
	Pig CD1e	ALQVLEPRHPAAEEPLS
	Rabbit CD1e	GSEALRPHHPAAEEPLT

[a] The N-terminal sequences were predicted using http://www.cbs.dtu.dk/services/Signal/. The accession numbers of the aminoacid sequences of the human CD1a, b, c d and e proteins are NP_001754, NP_001755, CAG33361, AAA59673 and NP_112155, respectively. The accession numbers of the aminoacid sequences of macaque, pig, guinea-pig and rabbit CD1e are NP_001036123, NM_001034394, BAF38761, AAF12745 and AAG39380, respectively.

Table II

N- or C-terminal aminoacid sequences of human membrane-associated and soluble CD1e.

CD1e form	m/z value[a]	Mass[b]	Sequence[c]	Loc[d]
mCD1e	720.30(3+)	2158.08	APQALQSYHLAAEEQLSFR	N
mCD1e	597.93(3+)	1791.90	LQSYHLAAEEQLSFR	N
mCD1e	650.79(3+)	1299.66	HLAAEEQLSFR	N
mCD1e	525.72(3+)	1049.51	AAEEQLSFR	N
sCD1e	454.69(2+)	907.44	EEQLSFR	N
sCD1e	529.18(3+)	1584.78	HSSLFGGHDLIIHWGG	C

CD1e molecules were immunopurified from transfected cells and separated by SDS-PAGE (Fig. 2A). Tryptic fragments of the membrane-associated and soluble forms were analyzed by MALDI mass spectrometry (Fig 2B) and *de novo* sequencing after LC-MS/MS (Fig 2C).

[a] 2+ and 3+ indicate doubly-and triply- charged ions, respectively

[b] Monoisotopic molecular mass of the peptide

[c] Sequences were determined by *de novo* sequencing after LC-MS/MS (Fig. 2C)

[d] Localization of the sequences on CD1e molecule. N, N-terminus, C, C-terminus.

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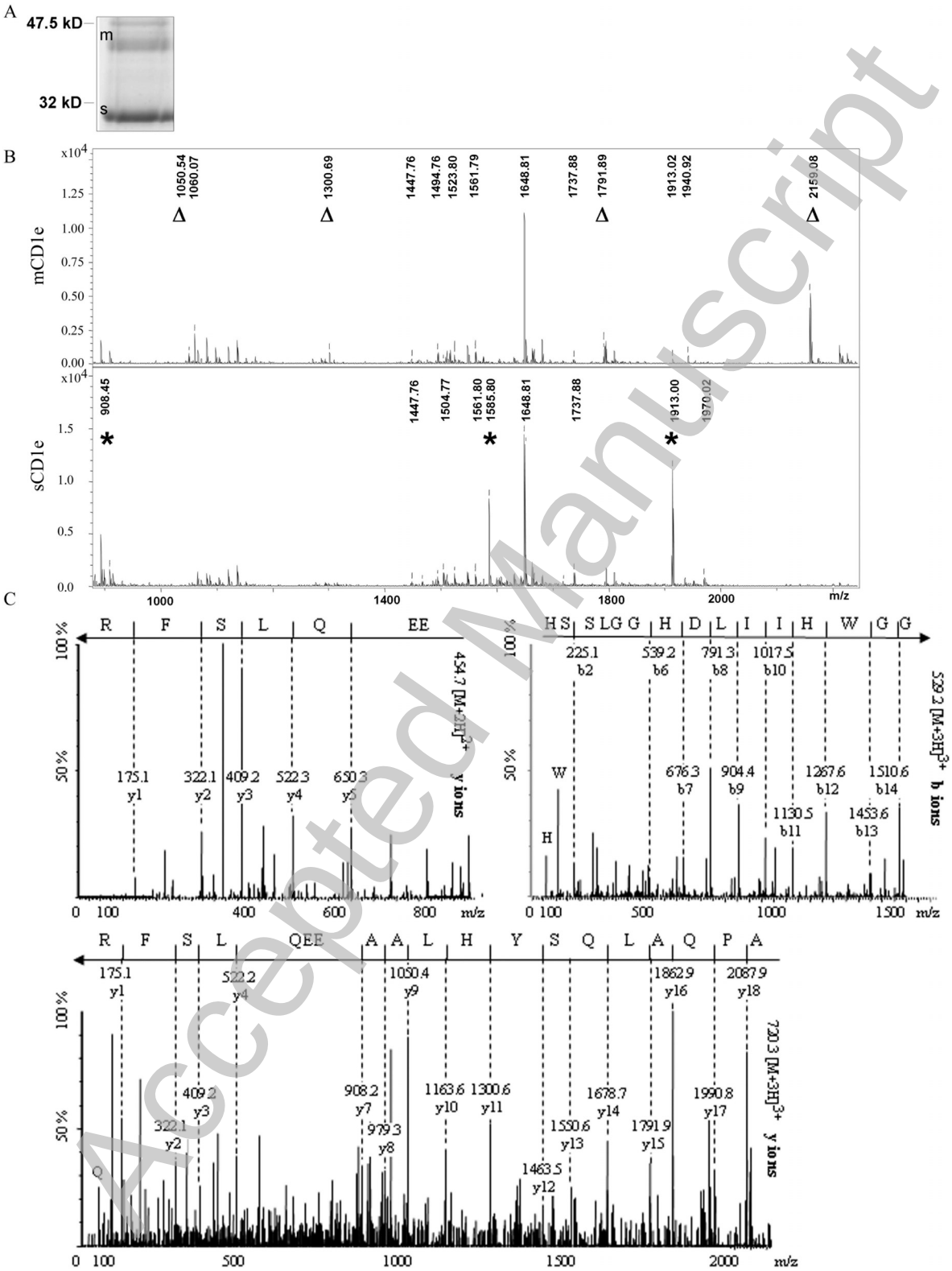

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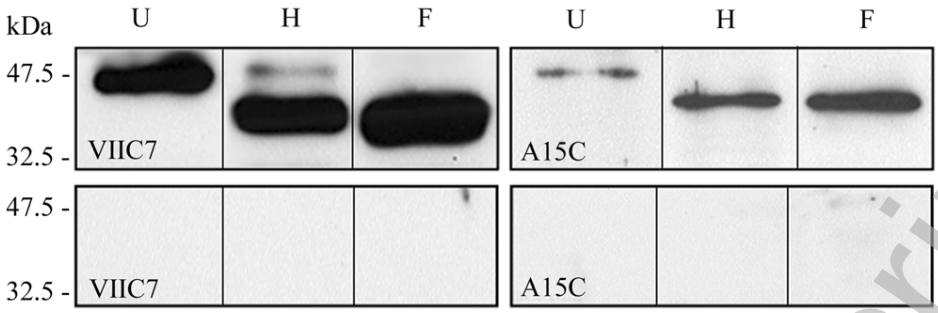

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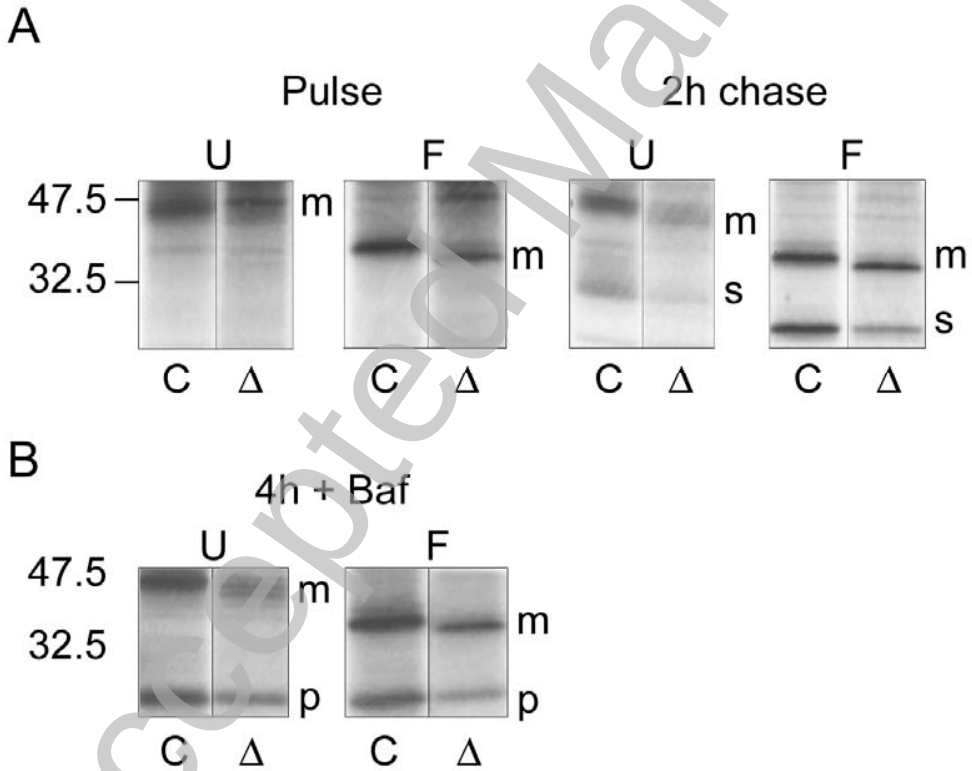

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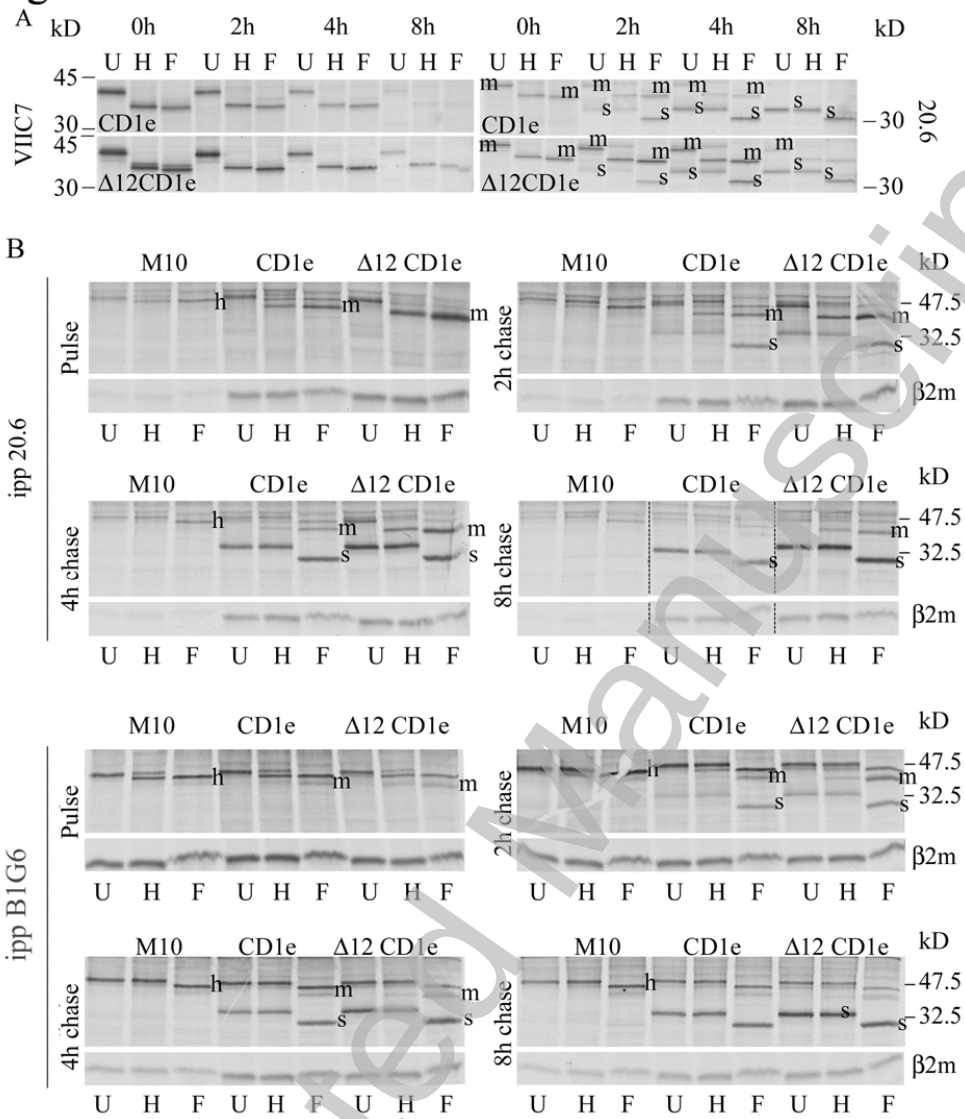
Figure 4



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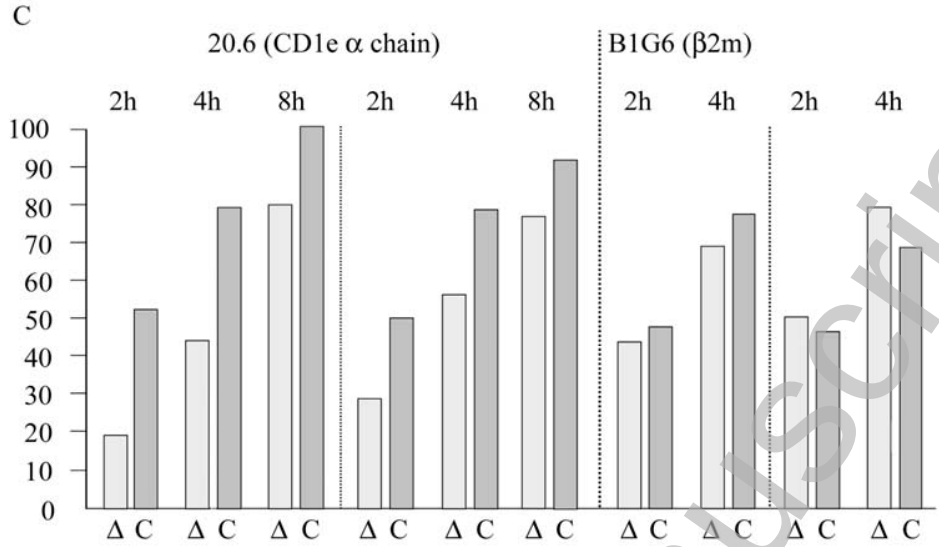

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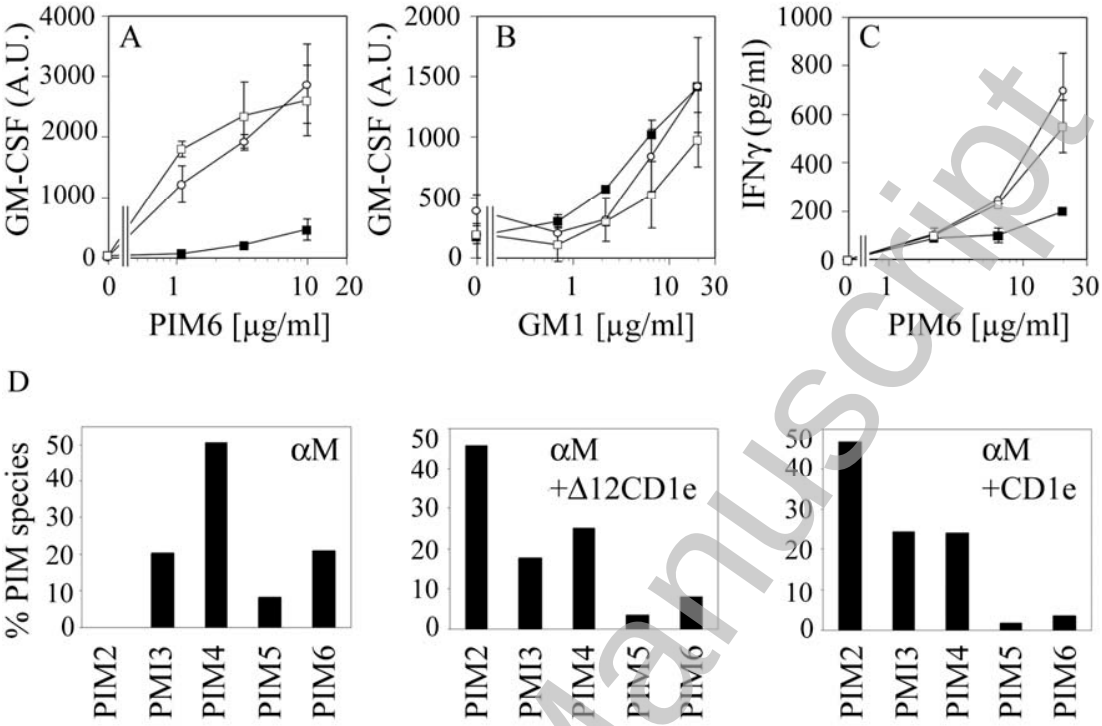

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